

**UNITED STATES PATENT APPLICATION**

**OF**

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**FOR**

**GENES REGULATING PROGRAMMED CELL DEATH**

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## GOVERNMENT RIGHTS IN INVENTION

Some aspects of the invention disclosed in this application were supported by the United States government, National Institute of Health Grant No. GM 59136. Accordingly, the U.S. Government has certain rights in the invention hereof.

## 5 CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit from U.S. Provisional Application Serial No. 60/243,865, filed October 27, 2000.

*now abandoned.*

## BACKGROUND OF THE INVENTION

### 10 Field of the Invention

The present invention relates generally to apoptosis or programmed cell death, and more particularly, to amino acid sequences of proteins that are associated with apoptosis and the use of these proteins in the diagnosis, prevention and treatment of disorders associated  
15 with abnormal cell proliferation and apoptosis.

### Background of the Related Art

Programmed cell death or apoptosis plays a critical role during animal development by  
20 functioning in the destruction of unneeded cells and tissues. The term programmed death was established to distinguish physiological or genetic-regulated cell death from necrotic cell destruction. Genetically regulated cell death is an integral component of normal development, and is used in processes such as, limb formation and nervous system remodeling. Cell death is also involved in removal of abnormal cells during  
25 development, including those during tumor genesis.

It has now become clear that disturbances in programmed cell death, which prevent or delay normal cell turnover, can be just as important to the pathogenesis of diseases as known abnormalities in the regulation of proliferation and the cell cycle. Like cell division, which is controlled through complex interactions between cell cycle regulatory proteins, programmed cell death is similarly regulated under normal circumstances by the interaction of gene products that either induce or inhibit cell death.

The stimuli that regulate the function of these apoptosis gene products include both extracellular and intracellular signals. Either the presence or the removal of a particular stimulus can be sufficient to evoke a positive or negative apoptosis signal. For example, physiological stimuli that prevent or inhibit apoptosis include growth factors, extracellular matrix, CD40 ligand, viral gene products, neutral amino acids, zinc, estrogen and androgens. In contrast, stimuli that promote apoptosis include growth factors such as tumor necrosis factor (TNF), Fas and transforming growth factor  $\beta$  (TGF  $\beta$ ). Other stimuli that promote apoptosis include, for example, neurotransmitters, growth factor withdrawal, loss of extracellular matrix attachment, intracellular calcium and glucocorticoids. Although apoptosis is mediated by diverse signals and complex interactions of cellular gene products, the results of these interactions ultimately feed into a cell death pathway that is evolutionarily conserved between humans and invertebrates.

Several gene products that modulate the apoptotic process have now been identified. Although these products can be generally separated into two basic categories, gene products from each category can function to either inhibit or induce programmed cell death. One family of gene products is related to the protein Bcl-2, which inhibits apoptosis when overexpressed in cells. A second family of gene products, the aspartate-specific cysteine proteases (ASCPs), are genetically related to the ced-3 gene product, which was initially shown to be required for programmed cell death in the roundworm, *C. elegans* (Ellis et al., 1991)

A series of elegant genetic studies of programmed cell death in the worm *C. elegans* led to the isolation of the *ced-3*, *ced-4* and *ced-9* genes. *Ced-3* is homologous to the mammalian family of caspases, which upon proteolytic activation are critical effectors of the programmed cell death signaling pathway (Cryns and Yuan, 1998). *CED-4* is  
5 homologous to mammalian Apaf-1, which activates caspases in the presence of cytochrome c and dATP. *CED-9* is a member of the Bcl-2 family of cell death regulators and found to represses the apoptosis of many different cells in the nematode development.

10 Apoptosis maintains tissue homeostasis in a range of physiological processes such as embryonic development, immune cell regulation and normal cellular turnover. Therefore, the dysfunction or loss of regulated apoptosis can lead to a variety of pathological disease states. Clearly, there is a need for factors that are useful for inducing apoptosis for therapeutic purposes, for example, as an antiviral agent, an anti-tumor agent to control  
15 embryonic development and tissue homeostasis, and the roles of such factors in dysfunction and disease. Further, there is a clear need for factors that are useful for reducing or halting apoptosis for therapeutic purposes, for example, to treat diseases caused or associated with apoptosis, such as Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, chronic inflammation, acute inflammation, AIDs, degenerative liver  
20 disease and the like. Treatments that are specifically designed to modulate the apoptotic pathways in these and other pathological conditions can change the natural progression of many of these diseases.

Genetic screens in *Drosophila* have revealed three cell death inducer genes: reaper (*rpr*)  
25 head involution defective (*hid*) and *grim*. The function of these genes is required for virtually all programmed cell death during embryogenesis. Ectopic expression of each of these genes is also sufficient to induce caspase-dependent programmed cell death in a wide range of cell types. Although vertebrates homologs of *rpr*, *hid* and *grim* have not yet been identified, expression of each of these genes is sufficient to induce apoptosis in

mammalian cells (McCarthy and Dixit, 1998; Haining et al., 1999) suggesting that the downstream death pathway may be conserved between flies and mammals.

The similarity between vertebrate and invertebrate cell death pathways establishes *Drosophila* as a valuable model system for genetics studies of the regulation of apoptosis in humans and thus could be the vehicle to identify new apoptotic genes and their gene products to modulate apoptosis for the therapeutic treatment of human diseases.

## SUMMARY OF THE INVENTION

The present invention relates to identifying novel proteins that possess apoptosis activity by homology between the amino acid sequence of SEQ ID NO: 1 and other amino acid sequences of proteins in both vertebrates and invertebrates. Included in the homologous amino acid sequences that possess apoptosis activity are SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8.

The present invention also relates to a protein associated with apoptosis having at least 60% homology over the complete sequence of the amino acid sequence of SEQ ID NO: 1.

The present invention further relates to identifying a modulator of apoptosis to ameliorate the effects of Alzheimer's disease, cancer, Parkinson's disease, rheumatoid arthritis, chronic inflammation, acute inflammation, AIDs, and degenerative liver disease.

The present invention also provides a method for preventing or treating a disorder associated with a decrease in apoptosis, the method comprising:

administering to a subject in need of such treatment an effective amount of a pharmaceutical composition comprising a apoptotically active protein having an amino acid sequence (i) of SEQ ID NO: 1 or (ii) with at least 60% homology to SEQ ID NO: 1.

7. The method according to claim 6, wherein the homologous amino acid sequence (ii) is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8.

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The homologous amino acid sequence comprising 60% amino acid identity over the complete sequence of SEQ ID NO: 1 is selected from the group consisting of SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO: 5 and SEQ ID NO 8.

10 This invention further provides for a purified and isolated nucleic acid molecule encoding a mutated protein with homology to SEQ ID NO: 1.

The present invention also provides a method for detecting a polynucleotide that encodes an apoptotic protein having at least one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8 in a biological test sample containing nucleic acids, the method comprising the steps of:

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(a) mixing at least a fragment of a complement of the polynucleotide sequence encoding at least a fragment of a protein having at least one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8 with the biological test sample containing nucleic acids, to form a resulting mixture;

(b) subjecting the mixture to conditions such that hybridization will occur between the biological test sample and the complement of the polynucleotide sequence encoding at least a fragment of a protein having at least one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8; and

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(c) detecting hybridization complexes in the mixture subjected to hybridization conditions, wherein the presence of a hybridization complex correlates with the presence of a polynucleotide encoding a protein having at least one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8 in the biological test sample.

Further, the nucleic acids of the biological test sample may be amplified by a polymerase chain reaction prior to hybridizing step.

In yet another aspect, the present invention relates to a method for screening a potential cellular apoptosis inhibiting compound for determining its utility as a therapeutic agent for treatment of diseases associated with increased programmed cell death, the method comprising:

- (a) contacting a cell which expresses a protein including at least one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8 with the test compound; and
- (b) determining the level of apoptosis activity of the cell, wherein a decrease in activity identifies a compound that inhibits apoptotic activity.

In another aspect, the present invention further provides an expression vector containing at least a fragment of a polynucleotide sequence encoding a polypeptide comprising at least one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8.

In another aspect this expression vector is contained within a host cell and transforms the host cell to express therapeutically effective amounts of a polypeptide comprising at least one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8.

This invention also provides a method for producing a polypeptide comprising at least one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8, the method comprising:

(a) culturing a host cell containing an expression vector containing a polynucleotide sequence encoding for a polypeptide comprising at least one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8 under conditions suitable for the expression of the polypeptide; and

(b) recovering the expressed polypeptide from the host cell line.

The present invention further relates to a purified antibody which binds to a polypeptide comprising at least one amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8, as well as an agonist and an antagonist to the polypeptides of the present invention.

The invention also relates to a method for preventing or treating a disorder associated with decreased apoptosis comprising administering to a subject in need of such treatment a pharmaceutical composition comprising a polynucleotide sequence that encodes for a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8 or a composition comprising a peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5 and SEQ ID NO: 8.

Other features and advantages of the invention will be apparent from the following detailed description, drawings and claims.



## BRIEF DESCRIPTION OF THE FIGURES

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A1 ✓  
5 Figure 1 shows the alignment of the amino acid domain that is conserved between *Drosophila* E93 and predicted proteins in humans, fish, mice and worms.

✓  
10 Figure 2 shows the nucleotide sequence of the human *E93A* (A) and *E93B* (B) transcription units.

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10 Figure 3 shows the genomic organization of the human *E93A* (A) and *E93B* (B) transcription units. The *hE93A* primary transcript is greater than 140 kilobases (kb) in length, while the *hE93B* primary transcript is 10 kb in length.

✓  
15 Figure 4 shows the amino acid sequence of the predicted human E93 protein. The conserved amino acid domain is underlined.

20 Figure 5 shows Northern blot hybridization analyses of human *E93* transcription in different tissues. Human *E93* transcripts were detected in brain, kidney, muscle, small intestine, and testis.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

### I. The Invention

25 Isolation of the correct sequence for the *Drosophila* E93 gene that encodes for a protein found to modulate apoptosis provided the tool for isolation of proteins associated with modulation of programmed cell death and the encoded proteins. The discovery of proteins with apoptosis activity can be as agonists, antagonists, antibodies and for treating disorders related to apoptosis.

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A proposed *Drosophila* E93 gene (SEQ ID NO: 12) was first identified based on its induction by the steroid 20-hydroxyecdysone (ecdysone) just prior to programmed cell death of larval salivary glands (Baehrecke and Thummel, 1995). At the time the first proposed E93 gene was isolated, no similar genes had been identified. However, subsequent to the isolation of the first proposed E93 gene an error was discovered in the original gene sequence that had a marked effect on the predicted E93 protein (SEQ ID NO: 11). Specifically, the omission of a nucleotide that changed the reading frame encoded for an amino acid sequence markedly different from that of the present invention. The amino acid residue sequence in SEQ ID NO: 11 ranging from 776 to and including 956 has since been determined to be incorrect.

The recently identified and corrected gene sequence (SEQ ID NO: 9) that encodes for an amino acid sequence (SEQ ID NO: 10 with a different amino acid residue sequence ranging from 775 to and including 955) has provided the present inventor with a tool to identify a 53 amino acid domain in *Drosophila* E93 (SEQ ID NO: 1) that is conserved in the human *H. sapiens* (SEQ ID NO: 2), the fish *T. nigroviridis* ((SEQ ID NO: 3), the mouse *M. musculus* (SEQ ID NO: 4) and the nematode *C. elegans*, (SEQ ID NO: 5) as shown in Figure 1.

Animals that possess mutations in E93 have defects in programmed cell death, and their salivary glands do not die (Lee and Baehrecke, 2001; Lee et al., 2000). E93 protein binds to chromosomes, and E93 mutants exhibit defects in cell death gene transcription including the caspase *dronc*. Furthermore, expression of E93 is sufficient to induce programmed cell death in different *Drosophila* cells types during development. Previously isolated steroid-regulated genes that function in programmed cell death also regulate cell differentiation and morphogenesis in *Drosophila*, while E93 appears to function more specifically in cell killing. Combined, these data indicate that *Drosophila* E93 regulates programmed cell death by regulating the transcription of programmed cell death genes.

1 The human *E93* (*hE93*) gene has been characterized and found to include two distinct  
RNAs based on the isolation of related but independent cDNAs. One cDNA was isolated  
from a human testis (Figure 2A) library and the other cDNA from a fetal brain (Figure  
2B) library that were obtained from Origene, Inc. These cDNAs were sequenced on both  
5 strands and are identical in most of their sequence, but possess different 5' ends. The  
cDNA isolated from testis encodes a 4958 base RNA that has been named *hE93A*, and the  
cDNA isolated from fetal brains encodes a 6074 base RNA that has been named *hE93B*.  
These sequences map to the same region of human chromosome 4, and are alternative  
transcript forms of *hE93*. The transcripts utilize alternative promoters and splicing, but  
10 encode identical predicted proteins. In Figure 2A, bases 1-690 encode the *hE93A*-  
specific region and in Figure 2B the *hE93B*-specific region includes bases 1-819. The  
remaining bases in both Figure 2A and 2B encode for the same polypeptide comprising a  
53 amino acid domain that is conserved between *Drosophila* E93 and the human *hE93*  
protein. Specifically, bases 1547 to 1705 in Figure 2A and bases 2676 to 2834 in Figure  
15 2B are conserved with the amino acid domain of *Drosophila* E93.

In order to facilitate review of the various embodiments of the invention and provide an  
understanding of the various elements and constituents used in making and using the  
present invention, the following terms used in the invention description have the  
20 following meanings.

## 2. Definition

An "allele" or "allelic sequence", as used herein, is an alternative form of the gene  
25 encoding for a polypeptide comprising the any one of the amino acid sequences of SEQ  
ID NOs: 1-5 and 8. Alleles may result from at least one mutation in the nucleic acid  
sequence and may result in altered mRNAs or polypeptides whose structure or function  
may or may not be altered. Any given natural or recombinant gene may have none, one,  
or many allelic forms. Common mutational changes which give rise to alleles are  
30 generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of

these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

"Altered" nucleic acid sequences encoding a polypeptide comprising any one of the amino acid sequences of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10, as used herein, include those with deletions, insertions, or substitutions of different nucleotides resulting in a polynucleotide that encodes the same or a functionally equivalent polypeptide. The encoded protein may also be "altered" and contain deletions, insertions, or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent polypeptide.

"Amino acid sequence", as used herein, refers to an oligopeptide, peptide, polypeptide, or protein sequence, and fragment thereof, and to naturally occurring or synthetic molecules. Fragments of a polypeptide comprising any one of the amino acid sequences of SEQ ID NOs: 1-5 and 8 are preferably about 15 to about 30 amino acids in length and retain the biological activity or the immunological activity of the full polypeptide.

"Amplification", as used herein, refers to the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction (PCR) technologies well known in the art (PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y.).

The term "antagonist", as used herein, refers to a molecule which, when bound to a polypeptide comprising any one of the amino acid sequences of SEQ ID NOs: 1-5 and 8, decreases the amount or the duration of the effect of the biological activity of polypeptide.

The term "agonist" as used herein, refers to a molecule which, when bound to a polypeptide comprising any one of the amino acid sequences of SEQ ID NOs: 1-5 and 8, increases or prolongs the effect of the polypeptide.

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As used herein, the term "antibody" refers to intact molecules as well as fragments thereof, such as Fa, F(ab')<sub>2</sub>, and Fv, which are capable of binding the epitopic determinant. Antibodies that bind a polypeptide comprising any one of the amino acid sequences of SEQ ID NOs: 1-5 can be prepared using intact polypeptides or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or oligopeptide used to immunize an animal can be derived from the translation of RNA or synthesized chemically and can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin and thyroglobulin, keyhole limpet hemocyanin. The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

The term "antigenic determinant", as used herein, refers to that fragment of a molecule (i.e., an epitope) that makes contact with a particular antibody. When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (i.e., the immunogen used to elicit the immune response) for binding to an antibody.

The term "biologically active", as used herein, refers to a protein having structural, regulatory, or biochemical functions of a naturally occurring molecule. Likewise, "immunologically active" refers to the capability of the natural, recombinant, or synthetic polypeptide comprising any one of the amino acid sequences of SEQ ID NOs: 1-5, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

The terms "complementary" or "complementarity", as used herein, refer to the natural binding of polynucleotides under permissive salt and temperature conditions by base-pairing. For example, the sequence "A-G-T" binds to the complementary sequence "T-C-A". Complementarity between two single-stranded molecules may be "partial", in which

only some of the nucleic acids bind, or it may be complete when total complementarity exists between the single stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands.

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A "composition comprising a given polynucleotide sequence", as used herein, refers broadly to any composition containing the given polynucleotide sequence. The composition may comprise a dry formulation or an aqueous solution. Compositions comprising polynucleotide sequences encoding polypeptide comprising any one of the amino acid sequences of SEQ ID NOs: 1-5 and 8 may be employed as hybridization probes. The probes may be stored in freeze-dried form and may be associated with a stabilizing agent such as a carbohydrate. In hybridizations, the probe may be deployed in an aqueous solution containing salts (e.g., NaCl), detergents (e.g., SDS) and other components (e.g., Denhardt's solution, dry milk, salmon sperm DNA, etc.).

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The term "correlates with expression of a polynucleotide", as used herein, indicates that the detection of the presence of ribonucleic acid that is similar to SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:9 by northern analysis is indicative of the presence of mRNA encoding a polypeptide comprising any one of the amino acid sequences of SEQ ID NOs: 1-5 and 8 or a homologous amino acid sequence with at least 60% homology and thereby correlates with expression of the transcript from the polynucleotide encoding the protein.

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A "deletion", as used herein, refers to a change in the amino acid or nucleotide sequence and results in the absence of one or more amino acid residues or nucleotides.

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The term "homology", as used herein, refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target

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sequence may be examined using a hybridization assay (Southern or northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or hybridization probe will compete for and inhibit the binding of a completely homologous sequence to the target sequence under conditions of low stringency.

The term "hybridization", as used herein, refers to any process by which a strand of nucleic acid binds with a complementary strand through base pairing.

10 The term "hybridization complex", as used herein, refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary G and C bases and between complementary A and T bases; these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (e.g., paper, membranes, filters, chips, pins or glass slides, or any other appropriate substrate to which cells or their nucleic acids have been fixed).

20 An "insertion" or "addition", as used herein, refers to a change in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively, as compared to the naturally occurring molecule.

"Microarray" refers to an array of distinct polynucleotides or oligonucleotides arranged on a substrate, such as paper, nylon or other type of membrane, filter, chip, glass slide, or any other suitable solid support.

The term "modulate", as used herein, refers to a change in the activity of a polypeptide comprising any one of the amino acid sequences of SEQ ID NOs: 1-5 and 8. For example, modulation may cause an increase or a decrease in protein activity, binding

characteristics, or any other biological, functional or immunological properties of the polypeptide.

"Nucleic acid sequence" as used herein refers to an oligonucleotide, nucleotide, or polynucleotide, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand.

The terms "specific binding" or "specifically binding", as used herein, refers to that interaction between a protein or peptide and an agonist, an antibody and an antagonist. The interaction is dependent upon the presence of a particular structure (i.e., the antigenic determinant or epitope) of the protein recognized by the binding molecule.

As used herein, the term "stringent conditions" refers to conditions which permit hybridization between polynucleotide sequences and the claimed polynucleotide sequences. Suitably stringent conditions can be defined by, for example, the concentrations of salt or formamide in the prehybridization and hybridization solutions, or by the hybridization temperature, and are well known in the art. In particular, stringency can be increased by reducing the concentration of salt, increasing the concentration of formamide, or raising the hybridization temperature.

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The term "purified", as used herein, refers to nucleic or amino acid sequences that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated.

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A "substitution", as used herein, refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

"Transformation", as defined herein, describes a process by which exogenous DNA enters and changes a recipient cell. It may occur under natural or artificial conditions using

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various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is selected based on the type of host cell being transformed and may include, but is not limited to, viral infection, electroporation, heat shock, lipofection, and particle bombardment. Such "transformed" cells include stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome. They also include cells which transiently express the inserted DNA or RNA for limited periods of time.

A "variant" of a polypeptide comprising any one of the amino acid sequences of SEQ ID NOs: 1-5, 8 and 10, as used herein, refers to an amino acid sequence that is altered by one or more amino acids. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Analogous minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTAR software.

## II. Polynucleotide

### A. Isolated and Purified Polynucleotides

In one aspect, the present invention provides for an isolated and purified polynucleotide sequence selected from the group consisting of SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 9. Additionally, the polynucleotides of the present invention may encode for a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10.

As used herein, the term "polynucleotide" means a sequence of nucleotides connected by phosphodiester linkages. Polynucleotides are presented herein in the direction from the 5' to the 3' direction. A polynucleotide of the present invention can be a deoxyribonucleic acid (DNA) molecule or ribonucleic acid (RNA) molecule. Where a polynucleotide is a DNA molecule, that molecule can be a gene or a cDNA molecule. Nucleotide bases are indicated herein by a single letter code: adenine (A), guanine (G), thymine (T), cytosine (C), inosine (I) and uracil (U).

The invention also encompasses a variant of a polynucleotide sequence encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10. In particular, such a variant polynucleotide sequence will have at least about 80%, more preferably at least about 90%, and most preferably at least about 95% polynucleotide sequence identity to a polynucleotide sequence encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10.

A polynucleotide of the present invention can be prepared using standard techniques well known to one of skill in the art. The preparation of a cDNA molecule encoding a peptide of the present invention is described hereinafter. A polynucleotide can also be prepared from genomic DNA libraries using lambda phage technologies.

In another aspect, the present invention provides an isolated and purified polynucleotide that encodes a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10, where the polynucleotide is preparable by a process comprising the steps of constructing a library of cDNA clones from a cell that expresses the polypeptide; screening the library with a labeled cDNA probe prepared

from RNA that encodes the polypeptide; and selecting a clone that hybridizes to the probe.

## B. Probes and Primers

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In another aspect, DNA sequence information provided by the present invention allows for the preparation of relatively short DNA (or RNA) sequence having the ability to specifically hybridize to gene sequences of the selected polynucleotide disclosed herein. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of a selected nucleotide sequence. The ability of such nucleic acid probes to specifically hybridize to a polynucleotide encoding a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10 lends them particular utility in a variety of embodiments. Most importantly, the probes can be used in a variety of assays for detecting the presence of complementary sequences in a given sample.

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To provide certain of the advantages in accordance with the present invention, a preferred nucleic acid sequence employed for hybridization studies or assays includes probe molecules that are complementary to at least a 10 to 70 or so long nucleotide stretch of a polynucleotide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10. A size of at least 10 nucleotides in length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 10 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 25 to 55. Such fragments can be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of

nucleic acid reproduction technology, such as the PCR or by excising selected DNA fragments from recombinant plasmids containing appropriate inserts and suitable restriction enzyme sites.

- 5 Accordingly, a polynucleotide probe molecule of the invention can be used for its ability to selectively form duplex molecules with complementary stretches of the gene. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degree of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one will typically desire
- 10 to employ relatively stringent conditions to form the hybrids. For example, one will select relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M NaCl at temperatures of 50°C to 70°C. Those conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.
- 15 Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate a peptide coding sequence from other cells, functional equivalents, or the like, less stringent hybridization conditions are typically needed to allow formation of the heteroduplex. In these circumstances, one can desire to employ conditions such as
- 20 0.15M-0.9M salt, at temperatures ranging from 20°C to 70°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus,
- 25 hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it is advantageous to employ a polynucleotide of the present invention in combination with an appropriate label for detecting hybrid formation. A

wide variety of appropriate labels are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal.

In general, it is envisioned that a hybridization probe described herein is useful both as a reagent in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions depend as is well known in the art on the particular circumstances and criteria required (e.g., on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe). Following washing of the matrix to remove non-specifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

### III. Peptides and Gene Transcription Regulatory Peptide

In one embodiment, the present invention contemplates an isolated and purified peptide that modulates programmed cell death. Preferably, the peptide that modulates programmed cell death has at least 60% homology to a peptide comprising an amino acid sequence of SEQ ID NO: 1 including at least one amino acid sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10.

The invention also encompasses peptide variants. A preferred variant is one which has at least about 80%, more preferably at least about 90%, and most preferably at least about 95% amino acid sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10, and which modulates programmed cell death.

Polypeptides are disclosed herein as amino acid residue sequences. Those sequences are written left to right in the direction from the amino to the carboxyl terminus. In accordance with standard nomenclature, amino acid residue sequences are denominated by either a single letter or a three letter code as indicated below.

5

	Amino Acid Residue	3-Letter Code	1-Letter Code
	Alanine	Ala	A
	Arginine	Arg	R
	Asparagine	Asn	N
10	Aspartic Acid	Asp	D
	Cysteine	Cys	C
	Glutamine	Gln	Q
	Glutamic Acid	Glu	E
	Glycine	Gly	G
15	Histidine	His	H
	Isoleucine	Ile	I
	Leucine	Leu	L
	Lysine	Lys	K
	Methionine	Met	M
20	Phenylalanine	Phe	F
	Proline	Pro	P
	Serine	Ser	S
	Threonine	Thr	T
	Tryptophan	Trp	W
25	Tyrosine	Tyr	Y
	Valine	Val	V

Modifications and changes can be made in the structure of a polypeptide of the present invention and still obtain a molecule having like peptide characteristics that modulate programmed cell death. For example, certain amino acids can be substituted for other amino acids in a sequence without appreciable loss of peptide activity. Because it is the interactive capacity and nature of a polypeptide that defines that polypeptide's biological functional activity, certain amino acid sequence substitutions can be made in a polypeptide sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a polypeptide with like properties.

In making such changes, the hydropathic index of amino acids can be considered. The importance of the hydropathic amino acid index in conferring interactive biologic

function on a polypeptide is generally understood in the art (Kyte, J. and R. F. Doolittle 1982). It is known that certain amino acids can be substituted for other amino acids having a similar hydropathic index or score and still result in a polypeptide with similar biological activity. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. Those indices are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is believed that the relative hydropathic character of the amino acid determines the secondary structure of the resultant polypeptide, which in turn defines the interaction of the polypeptide with other molecules, such as enzymes, substrates, receptors, antibodies, antigens, and the like. It is known in the art that an amino acid can be substituted by another amino acid having a similar hydropathic index and still obtain a functionally equivalent polypeptide. In such changes, the substitution of amino acids whose hydropathic indices are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine (See Table 1, below). The present invention thus contemplates functional or biological equivalents of a peptide as set forth above.

TABLE 1

<u>Original Residue</u>	<u>Exemplary Substitutions</u>
-------------------------	--------------------------------

	Ala	Gly; Ser
	Arg	Lys
	Asn	Gln; His
	Asp	Glu
5	Cys	Ser
	Gln	Asn
	Glu	Asp
	Gly	Ala
	His	Asn; Gln
10	Ile	Leu; Val
	Leu	Ile; Val
	Lys	Arg
	Met	Leu; Tyr
	Ser	Thr
15	Thr	Ser
	Trp	Tyr
	Tyr	Trp; Phe
	Val	Ile; Leu

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20 Biological or functional equivalents of a polypeptide can also be prepared using site-specific mutagenesis. Site-specific mutagenesis is a technique useful in the preparation of second generation polypeptides, or biologically functional equivalent polypeptides or peptides, derived from the sequences thereof, through specific mutagenesis of the underlying DNA. As noted above, such changes can be desirable where amino acid

25 substitutions are desirable. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as

30 well as a sufficient number of adjacent nucleotides, to provide a primer sequence of



sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed.

In general, the technique of site-specific mutagenesis is well known in the art. As will be appreciated, the technique typically employs a phage vector which can exist in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are commercially available and their use is generally known to those of skill in the art.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector which includes within its sequence a nucleotide sequence which encodes all or a portion of the peptide sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, by methods well known in the art. This primer is then annealed to the singled-stranded vector, and extended by the use of enzymes such as E. coli polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells such as E. coli cells and clones are selected which include recombinant vectors bearing the mutation. Commercially available kits come with all the reagents necessary, except the oligonucleotide primers.

A polypeptide of the present invention is prepared by standard techniques well known to those skilled in the art. Such techniques include, but are not limited to, isolation and purification from tissues known to contain that polypeptide, and expression from cloned DNA that encodes such a polypeptide using transformed cells.

#### IV. Expression Vectors

In another embodiment, the present invention provides expression vectors comprising polynucleotides that encode for modulating peptides of programmed cell death. Preferably, expression vectors of the present invention comprise polynucleotides that

5 encode polypeptides comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10. The expression vector can include the peptide coding region itself of any of the above sequences or it can contain coding regions bearing selected alterations or modifications in the basic coding region of such peptides.

10 Alternatively, such vectors or fragments can code larger polypeptides or peptides which nevertheless include the basic coding region. In any event, it should be appreciated that due to codon redundancy as well as biological functional equivalence, this aspect of the invention is not limited to the particular DNA molecules corresponding to the polypeptide sequences noted above.

15 The expression vectors of the present invention preferably comprise polynucleotides operatively linked to an enhancer-promoter. More preferably still, expression vectors of the invention comprise a polynucleotide operatively linked to a prokaryotic or eukaryotic promoter.

20 A promoter is a region of a DNA molecule typically within about 100 nucleotide pairs in front of (upstream of) the point at which transcription begins (i.e., a transcription start site). That region typically contains several types of DNA sequence elements that are located in similar relative positions in different genes. As used herein, the term

25 "promoter" includes what is referred to in the art as an upstream promoter region, a promoter region or a promoter of a generalized eukaryotic RNA Polymerase II transcription unit.

30 Another type of discrete transcription regulatory sequence element is an enhancer. An enhancer provides specificity of time, location and expression level for a particular

encoding region (e.g., gene). A major function of an enhancer is to increase the level of transcription of a coding sequence in a cell that contains one or more transcription factors that bind to that enhancer. Unlike a promoter, an enhancer can function when located at variable distances from transcription start sites so long as a promoter is present.

5

As used herein, the phrase "enhancer-promote" means a composite unit that contains both enhancer and promoter elements. An enhancer-promoter is operatively linked to a coding sequence that encodes at least one gene product. As used herein, the phrase "operatively linked" means that an enhancer-promoter is connected to a coding sequence in such a way that the transcription of that coding sequence is controlled and regulated by that enhancer-promoter. Means for operatively linking an enhancer-promoter to a coding sequence are well known in the art. As is also well known in the art, the precise orientation and location relative to a coding sequence whose transcription is controlled, is dependent inter alia upon the specific nature of the enhancer-promoter. Thus, a TATA box minimal promoter is typically located from about 25 to about 30 base pairs upstream of a transcription initiation site and an upstream promoter element is typically located from about 100 to about 200 base pairs upstream of a transcription initiation site. In contrast, an enhancer can be located downstream from the initiation site and can be at a considerable distance from that site.

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An enhancer-promoter used in a vector construct of the present invention can be any enhancer-promoter that drives expression in a cell to be transfected. By employing an enhancer-promoter with well-known properties, the level and pattern of gene product expression can be optimized. A coding sequence of an expression vector is operatively linked to a transcription terminating region. RNA polymerase transcribes an encoding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA). Transcription-terminating regions are well known

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in the art. A preferred transcription-terminating region is derived from a bovine growth hormone gene.

Exemplary vectors include the mammalian expression vectors of the pCMV family including pCMV6b and pCMV6c (Chiron Corp., Emeryville Calif.) and pRc/CMV (Invitrogen, San Diego, Calif.). In certain cases, and specifically in the case of these individual mammalian expression vectors, the resulting constructs can require co-transfection with a vector containing a selectable marker such as pSV2neo.

Where expression of recombinant polypeptide of the present invention is desired and a eukaryotic host is contemplated, it is most desirable to employ a vector, such as a plasmid, that incorporates a eukaryotic origin of replication. Additionally, for the purposes of expression in eukaryotic systems, one desires to position the desired peptide encoding sequence adjacent to and under the control of an effective eukaryotic promoter such as promoters used in combination with Chinese hamster ovary cells. To bring a coding sequence under control of a promoter, whether it is eukaryotic or prokaryotic, what is generally needed is to position the 5' end of the translation initiation side of the proper translational reading frame of the polypeptide between about 1 and about 50 nucleotides 3' of or downstream with respect to the promoter chosen. Furthermore, where eukaryotic expression is anticipated, one would typically desire to incorporate into the transcriptional unit, an appropriate polyadenylation site.

The pRc/CMV vector (available from Invitrogen) is an exemplary vector for expressing a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10 in mammalian cells, particularly COS, CHO, human MCF-F, human 293T and BHK bovine cells. A polypeptide of the present invention under the control of a CMV promoter can be efficiently expressed in mammalian cells. The pCMV plasmids are a series of mammalian expression vectors of particular utility in the present

invention. The vectors are designed for use in essentially all cultured cells and have been successfully expressed in simian COS cells, mouse L cells, CHO cells, and HeLa cells.

#### V. Transfected Cells

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In yet another embodiment, the present invention provides recombinant host cells transformed or transfected with a polynucleotide that encodes a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10, as well as transgenic cells derived from those transformed or transfected cells.

10

Means of transforming or transfecting cells with exogenous polynucleotide such as DNA molecules are well known in the art and include techniques such as calcium-phosphate- or DEAE-dextran-mediated transfection, protoplast fusion, electroporation, liposome mediated transfection, direct microinjection and adenovirus infection.

15

The most widely used method is transfection mediated by either calcium phosphate or DEAE-dextran. Although the mechanism remains obscure, it is believed that the transfected DNA enters the cytoplasm of the cell by endocytosis and is transported to the nucleus. Depending on the cell type, up to 90% of a population of cultured cells can be transfected at any one time. Because of its high efficiency, transfection mediated by calcium phosphate or DEAE-dextran is the method of choice for experiments that require transient expression of the foreign DNA in large numbers of cells. Calcium phosphate-mediated transfection is also used to establish cell lines that integrate copies of the foreign DNA, which are usually arranged in head-to-tail tandem arrays into the host cell genome.

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In the protoplast fusion method, protoplasts derived from bacteria carrying high numbers of copies of a plasmid of interest are mixed directly with cultured mammalian cells. After fusion of the cell membranes (usually with polyethylene glycol), the contents of the bacteria are delivered into the cytoplasm of the mammalian cells and the plasmid DNA is

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transported to the nucleus. Protoplast fusion is not as efficient as transfection for many of the cell lines that are commonly used for transient expression assays, but it is useful for cell lines in which endocytosis of DNA occurs inefficiently. Protoplast fusion frequently yields multiple copies of the plasmid DNA tandemly integrated into the host  
5 chromosome.

The application of brief, high-voltage electric pulses to a variety of mammalian and plant cells leads to the formation of nanometer-sized pores in the plasma membrane. DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of  
10 the redistribution of membrane components that accompanies closure of the pores. Electroporation can be extremely efficient and can be used both for transient expression of cloned genes and for establishment of cell lines that carry integrated copies of the gene of interest. Electroporation, in contrast to calcium phosphate-mediated transfection and protoplast fusion, frequently gives rise to cell lines that carry one, or at most a few,  
15 integrated copies of the foreign DNA.

Liposome transfection involves encapsulation of DNA or RNA within liposomes, followed by fusion of the liposomes with the cell membrane. The mechanism of how nucleotides is delivered into the cell is unclear but transfection efficiencies can be as high  
20 as 90%.

A transfected cell can be prokaryotic or eukaryotic. In general, prokaryotes are preferred for the initial cloning of nucleotides sequences and constructing the vectors useful in the invention. For example, E. coli K12 strains can be particularly useful. Other microbial  
25 strains which can be used include E. coli B, and E. coli X1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The  
30 vector ordinarily carries a replication site, as well as marking sequences which are

capable of providing phenotypic selection in transformed cells. For example, *E. coli* can be transformed using pBR322, a plasmid derived from an *E. coli* species. pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage  
5 must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own polypeptides.

Those promoters most commonly used in recombinant DNA construction include the  $\beta$ -lactamase (penicillinase) and lactose promoter systems and a tryptophan (TRP) promoter  
10 system. While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to introduce functional promoters into plasmid vectors.

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15 In addition to prokaryotes, eukaryotic microbes, such as yeast can also be used. *Saccharomyces cerevisiae* or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used. This plasmid already contains the *trp1* gene which provides a selection marker for a  
20 mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1. The presence of the *trp1* lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Suitable promoter sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes such as enolase,  
25 glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also introduced into the expression vector downstream from the  
30 sequences to be expressed to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the  
5 maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin or replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms can also be used as hosts. In principle, any such cell culture is workable, whether from  
10 vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years. Examples of such useful host cell lines are AtT-20, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COSM6, COS-1, COS-7, 293 and MDCK cell lines. Expression vectors for such cells ordinarily include (if  
15 necessary) an origin of replication, a promoter located upstream of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often  
20 derived from viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, Cytomegalovirus and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments can also be used, provided there is  
25 included the approximately 250 bp sequence extending from the HindIII site toward the BglII site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

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## VI. Preparation of Polypeptides

In yet another embodiment, the present invention contemplates a process of preparing a programmed cell death modulating peptide comprising transfecting cells with a polynucleotide that encodes a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10 to produce transformed host cells; and maintaining the transformed host cells under biological conditions sufficient for expression of the polypeptide. Preferably, the transformed host cells are eukaryotic cells.

Alternatively, the host cells are prokaryotic cells.

A host cell used in the process is capable of expressing a functional, recombinant peptide of the present invention. A variety of cells are amenable to a process of the invention, for instance, yeasts cells, human cell lines, and other eukaryotic cell lines well known to those of the art.

Following transfection, the cell is maintained under culture conditions for a period of time sufficient for expression of a peptide. Culture conditions are well known in the art and include ionic composition and concentration, temperature, pH and the like. Suitable medium for various cell types are well known in the art. In a preferred embodiment, temperature is from about 20 °C to about 50 °C. pH is preferably from about a value of 6.0 to a value of about 8.0. Other biological conditions needed for transfection and expression of an encoded protein are well known in the art.

Transfected cells are maintained for a period of time sufficient for expression of the desired peptide. A suitable time depends inter alia upon the cell type used and is readily determinable by a skilled artisan. Typically, maintenance time is from about 2 to about 14 days.

A recombinant peptide having the ability to modulate programmed cell death is recovered or collected either from the transfected cells or the medium in which the cells are cultured. Recovery comprises isolating and purifying the recombinant polypeptide. Isolation and purification techniques for polypeptides are well known in the art and  
5 include such procedures as precipitation, filtration, chromatography, electrophoresis and the like.

## VII. Antibodies

10 In still another embodiment, the present invention provides antibodies immunoreactive with a polypeptide of the present invention. The antibodies may include both monoclonal and polyclonal immunoreactive with a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10. Means for  
15 preparing and characterizing antibodies are well known in the art.

Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogen comprising a polypeptide or polynucleotide of the present invention, and collecting antisera from that immunized animal. A wide range of animal species can be used for the  
20 production of antisera. Typically an animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster or a guinea pig. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given polypeptide may vary in its immunogenicity. It is  
25 often necessary therefore to couple the immunogen (e.g., a polypeptide of the present invention) with a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and

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include glutaraldehyde, M maleimidobenzoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, immunogenicity to a particular immunogen can be enhanced by the use of non-specific stimulators of the immune response known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen used of the production of polyclonal antibodies varies inter alia, upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies is monitored by sampling blood of the immunized animal at various points following immunization. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored.

Typically, a monoclonal antibody of the present invention can be readily prepared by a technique which involves first immunizing a suitable animal with a selected antigen (e.g., a polypeptide of the present invention) in a manner sufficient to provide an immune response. Rodents such as mice and rats are preferred animals. Spleen cells from the immunized animal are then fused with cells of an immortal myeloma cell. Where the immunized animal is a mouse, a preferred myeloma cell is a murine NS-1 myeloma cell.

The fused spleen/myeloma cells are cultured in a selective medium to select fused spleen/myeloma cells from the parental cells. Fused cells are separated from the mixture of non-fused parental cells, for example, by the addition of agents that block the de novo synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block de novo synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with

hypoxanthine and thymidine as a source of nucleotides. Where azaserine is used, the media is supplemented with hypoxanthine. This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microliter plates, followed by testing the individual clonal supernatants for reactivity with an antigen-polypeptides. The selected clones can then be propagated indefinitely to provide the monoclonal antibody.

By way of specific example, to produce an antibody of the present invention, mice are injected intraperitoneally with between about 1-200  $\mu$ g of an antigen comprising a polypeptide of the present invention. B lymphocyte cells are stimulated to grow by injecting the antigen in association with an adjuvant such as complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed Mycobacterium tuberculosis). At some time (e.g., at least two weeks) after the first injection, mice are boosted by injection with a second dose of the antigen mixed with incomplete Freund's adjuvant. A few weeks after the second injection, mice are tail bled and the sera titered by immunoprecipitation against radiolabeled antigen. Preferably, the process of boosting and titering is repeated until a suitable titer is achieved. The spleen of the mouse with the highest titer is removed and the spleen lymphocytes are obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately  $5 \times 10^7$  to  $2 \times 10^8$  lymphocytes.

Mutant lymphocyte cells known as myeloma cells are obtained from laboratory animals in which such cells have been induced to grow by a variety of well-known methods. Myeloma cells lack the salvage pathway of nucleotide biosynthesis. Because myeloma cells are tumor cells, they can be propagated indefinitely in tissue culture, and are thus denominated immortal. Numerous cultured cell lines of myeloma cells from mice and rats, such as murine NS-1 myeloma cells, have been established.

Myeloma cells are combined under conditions appropriate to foster fusion with the normal antibody-producing cells from the spleen of the mouse or rat injected with the antigen/polypeptide of the present invention. Fusion conditions include, for example, the presence of polyethylene glycol. The resulting fused cells are hybridoma cells. Like  
5 myeloma cells, hybridoma cells grow indefinitely in culture. Hybridoma cells are separated from unfused myeloma cells by culturing in a selection medium such as HAT media (hypoxanthine, aminopterin, thymidine). Unfused myeloma cells lack the enzymes necessary to synthesize nucleotides from the salvage pathway because they are killed in the presence of aminopterin, methotrexate, or azaserine. Unfused lymphocytes also do not  
10 continue to grow in tissue culture. Thus, only cells that have successfully fused (hybridoma cells) can grow in the selection media. Each of the surviving hybridoma cells produces a single antibody. These cells are then screened for the production of the specific antibody immunoreactive with an antigen/polypeptide of the present invention. Single cell hybridomas are isolated by limiting dilutions of the hybridomas. The  
15 hybridomas are serially diluted many times and, after the dilutions are allowed to grow, the supernatant is tested for the presence of the monoclonal antibody. The clones producing that antibody are then cultured in large amounts to produce an antibody of the present invention in convenient quantity.

20 By use of a monoclonal antibody of the present invention, specific polypeptides of the invention can be recognized as antigens, and thus identified. Once identified, those polypeptides can be isolated and purified by techniques such as antibody-affinity chromatography. In antibody-affinity chromatography, a monoclonal antibody is bound to a solid substrate and exposed to a solution containing the desired antigen. The antigen  
25 is removed from the solution through an immunospecific reaction with the bound antibody. The polypeptide is then easily removed from the substrate and purified.

#### VIII. Pharmaceutical Compositions

In a preferred embodiment, the present invention provides pharmaceutical compositions comprising a programmed cell death modulating peptide and a physiologically acceptable carrier. More preferably, a pharmaceutical composition comprises a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1,  
5 SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10.

A composition of the present invention is typically administered parenterally in dosage unit formulations containing standard, well-known nontoxic physiologically acceptable  
10 carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes intravenous, intramuscular, intraarterial injection, or infusion techniques.

Injectable preparations, for example sterile injectable aqueous or oleaginous suspensions, are formulated according to the known art using suitable dispersing or wetting agents and  
15 suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

Among the acceptable vehicles and solvents that may be employed are water, Ringer's  
20 solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

Preferred carriers include neutral saline solutions buffered with phosphate, lactate, Tris,  
25 and the like. Of course, one purifies the vector sufficiently to render it essentially free of undesirable contaminants, such as defective interfering adenovirus particles or endotoxins and other pyrogens such that it does not cause any untoward reactions in the individual receiving the vector construct. A preferred means of purifying the vector involves the use  
30 of buoyant density gradients, such as cesium chloride gradient centrifugation.

A transfected cell can also serve as a carrier. By way of example, a liver cell can be removed from an organism, transfected with a polynucleotide of the present invention using methods set forth above and then the transfected cell returned to the organism (e.g. injected intravascularly).

## IX. Screening Assays

In yet another aspect, the present invention contemplates a process of screening substances for their ability to interact with a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10, the process comprising the steps of providing a polypeptide of the present invention and testing the ability of selected test substances to interact with that polypeptide.

The methods of the present invention make it possible to produce large quantities of a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10 for use in screening assays. More important, however, is the relative purity of the peptides provided by the present invention. A relatively pure peptide preparation for assaying a protein-protein interaction makes it possible to use elutive methods without invoking competing, and unwanted, side-reactions.

Screening assays of the present invention generally involve determining the ability of a candidate test substance to bind to the polypeptides of the present invention. The peptides of the present invention can be coupled to a solid support. The solid support can be agarose beads, polyacrylamide beads, polyacrylic beads or other solid matrices capable of being coupled to proteins. Well known coupling agents include cyanogen bromide, carbonyldiimidazole, tosyl chloride, and glutaraldehyde.

## X. Therapeutics

In cancers where there is an increase in cell proliferation, it may be desirable to increase the expression of a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10 to limit cell proliferation. Therefore, in one embodiment, a programmed cell death modulating peptide may be administered to a subject to prevent or treat cancer including, but not limited to, adenocarcinoma, leukemia, lymphoma, melanoma, myeloma, sarcoma, and teratocarcinoma, and, in particular, cancers of the adrenal gland, bladder, bone, bone marrow, brain, breast, cervix, gall bladder, ganglia, gastrointestinal tract, heart, kidney, liver, lung, muscle, ovary, pancreas, parathyroid, penis, prostate, salivary glands, skin, spleen, testis, thymus, thyroid, and uterus.

In disorders associated with an increase in cell death or apoptosis it may be desirable to decrease the activity of the peptides of the present invention by administering an antagonist of a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10 to stimulate cell proliferation. In particular, the antagonist may be added to a cell or cells in vivo using delivery mechanisms such as liposomes, viral based vectors, or electroinjection for the purpose of promoting regeneration or cell differentiation of the cell. Diseases include AIDS and other infectious or genetic immunodeficiencies; neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, retinitis pigmentosa, and cerebellar degeneration; myelodysplastic syndromes, such as aplastic anemia; ischemic injuries, such as myocardial infarction, stroke, and reperfusion injury; toxin-induced diseases, such as alcohol-induced liver damage, cirrhosis, and lathyrism; wasting diseases, such as cachexia; viral infections, such as those caused by hepatitis B and C, and osteoporosis.



An antagonist of the polypeptides of the present invention which comprise an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8 and/or SEQ ID NO: 10 may be produced using methods which are generally known in the art. In particular, polypeptides of the present invention may be used to produce antibodies or to screen libraries of pharmaceutical agents to identify those which specifically bind to these polypeptides of the present invention and reduce their apoptotic activity.

In other embodiments, any of the proteins, antagonists, antibodies, agonists, complementary sequences, or vectors of the invention may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy may be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

It is understood that modification that do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

#### EXAMPLE 1

##### Tissue Distribution

For further characterization of the hE93 A and B genes, analysis of tissue distribution was performed. This analysis was performed by RNA blot analysis with RNA isolated from human tissue. Briefly, tissue distribution of hE93 mRNA was performed on northern

blots prepared and obtained from Origene, Inc. Radioactive hE93 probe was prepared and the blots were hybridized with the common region of the *hE93* A and B transcription units, washed and then visualized by autoradiography.

It was found that *hE93* is transcribed at high levels in testis, and lower levels in brain, kidney, muscle, and small intestine, as shown in Figure 5. Two transcripts were detected that were approximately 5.0 and 7.0 kilobases in length. The smaller 5.0 kilobase transcript, found to be very abundant in testis, is similar in size to the cDNA that was isolated from a testis cDNA library, indicating that we have characterized a full length or nearly full length gene. While the 6074 base cDNA that was isolated from the brain library is smaller than the large transcript detected by northern blot hybridization, this cDNA has multiple stop codons in all three reading frames upstream of the start AUG codon that is shared by both cDNAs, indicating the identification of the full length hE93 protein.

## EXAMPLE 2

Expression of hE93 is sufficient to induce programmed cell death in human cells

The open reading region of the hE93 gene was placed into a tissue culture cell transfection vector such that hE93 would be expressed in cells to test if expression of the hE93 expressed protein is sufficient to induce programmed cell death. As controls, cells were transfected with an empty vector, the same vector except that it contains either: (1) green fluorescent protein (GFP) which is used to monitor transfection rate, (2) the proapoptotic protein Bax which is sufficient to induce programmed cell death (Adams and Cory, 1998), (3) the antiapoptotic protein Bcl-x1 (Adams and Cory, 1998). Each of these gene constructs were transfected into equal numbers of Bovine BHK, human MCF-7, and human 293T cell lines. Nineteen hours post transfection the cell viability was assayed (Table 1). The empty vector and GFP constructs did not significantly impact cell viability. In contrast, expression of Bax significantly reduced the viability of all 3 cell

lines as has been previously demonstrated for this proapoptotic protein. The antiapoptotic protein Bcl-xl had some impact on the viability of BHK and 293T cells. Interestingly, hE93 protein expression was the most potent inducer of cell death in the human MCF-7 and 293T cell lines, but did not induce significant levels of cell death in the bovine cells. This specificity is very unique. The data indicate that expression of the hE93 gene is sufficient to induce apoptosis.

Table 1.  
Percent cell viability when cell lines are transformed with expression constructs

TRANSFORMATION CONSTRUCTS					
cell line	empty vector	GFP	BAX	Bcl-xl	hE93
BHK	98	92	32	55	72
MCF-7	97	94	7	105	2
293-T	98	90	21	75	4

## References

All publications mentioned herein are incorporated herein by reference for the all purposes.

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